Pestic. Sci. 1997, **49**, 164–168

Enhancement of Carbofuran Degradation by Soil Enrichment Cultures, Bacterial Cultures and by Synergistic Interaction among Bacterial Cultures

Soudamini Mohapatra and M. D. Awasthi

Pesticide Residue Laboratory, Indian Institute of Horticultural Research, Hessaraghatta Lake PO, Bangalore-560089, India

(Received 4 April 1996; revised version received 8 August 1996; accepted 8 October 1996)

Abstract: Suspensions of soil repeatedly treated with carbofuran under glasshouse conditions enhanced the degradation of carbofuran significantly in a mineral salts medium. Ninety-seven per cent of the applied carbofuran degraded in a medium inoculated with soil suspensions treated with carbofuran, compared to 15% in uninoculated medium in 10 days. Out of the seven bacterial cultures isolated from the enrichment culture, two cultures identified as *Pseudomonas stutzeri* and *Bacillus pumilis* enhanced carbofuran degradation, resulting in more than 98% loss of the applied carbofuran in 30 days. The other cultures enhanced the degradation up to 70% within the same period. The mixture of all the seven cultures, however interacted synergistically, enhancing the degradation of carbofuran residues to 96% in 10 days.

Key words: carbofuran, insecticide, nematocide, synergistic interaction

1 INTRODUCTION

Carbofuran is an economically important insecticide and nematocide used in India mostly in soil for control of scale insects and root knot nematodes in horticultural crops.[1,2] There is considerable evidence indicating involvement of micro-organisms in the degradation of carbofuran.[3] Soil suspensions from flooded soil re-treated with carbofuran showed a significant capacity to degrade carbofuran in a nitrogen-free mineral salts medium.[4] Carbofuran degradation can be both chemical and microbial. In flooded soil the degradation is mainly by chemical hydrolysis,[5] but in nonflooded soil with regular irrigation degradation can be both chemical and microbial. There are a number of reports on the accelerated biodegradation of carbofuran in carbofuran re-treated soils.[6–8] An attempt was therefore made to study whether soil suspensions from dry soil previously treated with carbofuran accelerate its degradation and whether any synergistic interaction exists between pure cultures of bacteria in enhancing the degradation of carbofuran.

2 MATERIALS AND METHODS

2.1 Degradation of carbofuran in soil

Sandy loam soil (sand 70%, clay 20%, organic matter 1·2%, pH 6·6) with a history of no previous use of carbofuran was collected from the experimental farm of IIHR Bangalore, India. Two sets of soils, 20 g each, were taken in test tubes (2·5 × 20 cm). One set of soils were sterilized three times at 20 psi. An aqueous solution of carbofuran (89·5% purity from Rallis India Ltd), was added to all the soil samples at the rate of 20 μg g^{-1}. Carbofuran residues from three replicates of both sterile and nonsterile soil samples (20 g) were

Pestic. Sci. 0031-613X/97/$09.00

extracted using phosphate buffer (K_2HPO_4, 4·925 + KH_2PO_4, 0·465 g litre^{-1}) + methanol (1 + 9 by volume). The samples were further cleaned up and analysed by GLC.[9]

2.2 Enrichment of carbofuran-degrading micro-organisms

Soil enrichment cultures were prepared by repeated additions of carbofuran to sandy loam soil in pots under glasshouse conditions. The pots were irrigated twice a week. Ten milligrams of a commercial formulation of carbofuran was repeatedly added to the soil at 10-day intervals. Ten days after the fifth addition, the contents in each pot were mixed thoroughly. A portion of the soil was mixed with sterile water and the resulting soil suspension was used as the soil-enrichment culture. Soil suspensions were prepared from another set of the same soil similarly incubated but not amended with carbofuran.

The ability of the soil enrichment culture to degrade carbofuran was tested in a medium as follows. A mineral medium [$MgSO_4.7H_2O$ 0·2, K_2HPO_4 0·1, $CaSO_4$ 0·04, $FeSO_4.7H_2O$ 0·002 g litre^{-1} in distilled water; pH 6·2] supplemented with carbofuran as a sole source of both carbon and nitrogen was used in the study. Carbofuran (20 μg ml^{-1}) in 0·5 ml of acetone was added aseptically to pre-sterilized 150-ml Erlenmeyer flasks. After 24 h of acetone evaporation at room temperature, 20-ml portions of the mineral medium were dispensed into the flasks and equilibrated with 0·1 ml of the carbofuran enrichment culture. In another set, the medium was inoculated with the soil suspension from soil not treated with carbofuran. Uninoculated medium served as control. After incubation at room temperature (25 $\pm$ 2°C), residues in triplicate samples from each treatment were solvent-extracted and analysed by GLC.

2.3 Isolation and identification of carbofuran-degrading bacteria

For isolation of bacteria, the mineral medium supplemented with carbofuran was inoculated with carbofuran enrichment culture from carbofuran re-treated soil. After 30 days of incubation, serial dilutions of the inoculated medium were plated on nutrient agar medium (peptone, 5; beef extract, 3; agar, 16; carbofuran, 0·20 g litre^{-1} in distilled water, pH 7·0). Seven colonies of bacteria appeared on the agar plate. The bacterial colonies were isolated and purified by several transfers on modified Wakimoto agar medium[10] based on their colour, structure and colony size. All the seven bacterial colonies appearing on the agar plate were tested for their ability to degrade carbofuran as a sole source of carbon and nitrogen in the mineral salts medium (as described in Section 2.2). The individual cultures were grown in sterile water and the sterile mineral salts medium (20 ml) containing 20 μg ml^{-1} carbofuran was inoculated with 0·2 ml of sterile water suspensions of all the bacterial isolates individually. Residues of carbofuran in triplicate samples from each treatment were solvent-extracted and analysed by GLC. The most efficient carbofuran-degrading bacteria were further purified and identified. The identification work was carried out by the Institute of Microbial Technology, Chandigarh, India.

In another experiment all the seven individual pure bacterial isolates were mixed together in sterile water. The ability of the bacterial cultures to degrade carbofuran in the mineral medium was studied as described above. The mineral medium was inoculated with 0·5 ml of the sterile water suspensions of the mixed cultures.

2.4 Gas-liquid chromatography (GLC)

In the studies with enrichment and pure cultures, the residues of carbofuran in triplicate samples were extracted from the medium with ethyl acetate (3 × 30 ml). The solvent extracts were pooled and concentrated by flash evaporation to 10 ml and dried over anhydrous sodium sulfate. The residues in the ethyl acetate fraction were analysed by injecting 1 μl portions into a gas chromatograph (Varian Model 3600) equipped with a thermionic specific detector (TSD) by using a 5% OV-101 stainless steel 2 m column. The operating parameters for carbofuran were: nitrogen, 30 ml min^{-1}; hydrogen, 4·5 ml min^{-1}; air, 175 ml min^{-1}; column temperature, 190°C; injector temperature, 220°C; detector temperature, 280°C. Carbofuran was separated at the retention time of 2·2 min under these conditions. Using this extraction and analysis method the recovery of carbofuran was found to be 95–99%.

3 RESULTS AND DISCUSSION

3.1 Carbofuran degradation by soil enrichment cultures

The studies on the degradation of carbofuran in soil showed reduced degradation of residues following the incubation of carbofuran in sterilized soil as there was recovery of 56·1% of applied carbofuran after 40 days against 21% recovery from unsterilized soil (Table 1). According to reports published earlier,[6–8,11] repeated applications of carbofuran to normal agricultural soils under flooded condition have led to a build-up of micro-organisms capable of degrading carbofuran. Therefore an experiment was carried out to study whether a similar build-up of micro-organisms occurs in soil with regular irrigation. This is normally the condi-

TABLE 1
Degradation of Carbofuran in Natural and Sterile Sandy Loam Soil

Incubation (days)	*Carbofuran recovered (%)*[a]	
	Natural soil	*Sterile soil*
0	88·0	88·0
5	76·1	83·5
10	60·7	77·3
20	43·5	65·2
40	21·0	52·1

[a] The soil was treated with carbofuran at the rate of 20 μg g^{-1}. The percentage recovery given is the average of three replicates.

tion of soil in which horticultural crops are grown. Carbofuran was repeatedly applied to sandy loam soil in pots under glasshouse conditions. Soil suspensions from this soil that had been treated with four applications of carbofuran or soil untreated with carbofuran were tested for their ability to degrade carbofuran in a mineral salts medium as a sole source of carbon and nitrogen.

Carbofuran disappeared faster from a medium inoculated with carbofuran enrichment cultures than from an uninoculated medium or a medium inoculated with a suspension from soil not treated with carbofuran (Table 2). However, after 10 days of inoculation at room temperature, 97% of the applied carbofuran degraded from the medium inoculated with enrichment cultures from carbofuran-treated soil compared to 49% from soil not treated with carbofuran before. By 15 days 98% of the applied carbofuran degraded from medium inoculated with enrichment cultures from carbofuran-treated soil, 65% from medium inoculated with cultures from untreated soil and 16% from uninoculated medium. The disappearance of 97% of applied carbofuran within 10 days indicates that the carbofuran enrichment culture is highly active in accelerating the degradation of carbofuran. In a similar study earlier,[5] enrichment cultures from carbofuran-amended flooded soils effected a more rapid degradation of carbofuran in a mineral salts medium than the enrichment cultures from unamended soil.

3.2 Carbofuran degradation by pure cultures of bacteria

Seven pure cultures of bacteria were isolated from the medium inoculated with soil suspensions from carbofuran-retreated soil. The ability of all the bacteria to degrade carbofuran individually and in combination as a sole source of carbon and nitrogen was studied in a mineral salts medium incubated at room temperature ($25 \pm 2°C$). The most dominant culture (49–69%) and another which constituted only 3–5% of the total population, degraded almost all applied carbofuran in 30 days (Table 3). These two cultures were identified as *Pseudomonas stutzeri* (Leh. + Neu.) Sijd. and *Bacillus pumilis*. Out of the five unidentified cultures, three cultures designated as B1 (17–26%), B2 (9–11%) and B3 (5–10%) also enhanced the degradation of carbofuran. In the initial 10 days comparatively faster degradation was observed in medium inoculated with the unidentified cultures (Table 3). Though the rate of degradation varied in case of all the five cultures (*P. stutzeri*, *B. pumilis*, B1, B2 and B3), by 20 days of incubation around 60% of applied carbofuran was lost from all the media while 46% was lost from medium inoculated with *B. pumilis*. However, after 30 days, almost complete degradation was noticed from medium inoculated with *B. pumilis* while only 1·4% remained in the medium in the case of *P. stutzeri*. In case of the other cultures 11–35% remained in the media after 30 days, while, in the uninoculated control, 75% carbofuran remained undegraded in the medium after 30 days. Very little carbofuran was lost from the media inoculated with the other two unidentified cultures. These two cultures did not enhance carbofuran degradation at all even after 30 days, (data not shown). Enhanced degradation of carbofuran by micro-organisms isolated from carbofuran-retreated flooded soil has also been reported earlier.[12]

TABLE 2
Degradation of Carbofuran in a Mineral Salts Medium Inoculated with Suspensions from Soil Untreated and Repeatedly Treated with Carbofuran

Incubation (days)	*Carbofuran recovered (%)*[a]		
		Inoculated with soil suspensions from	
	Uninoculated	*Carbofuran-untreated soil*	*Carbofuran-treated soil*
0	98·9	98·9	98·9
10	86·1	50·2	3·0
15	83·9	35·1	1·2

[a] The mineral salts medium was supplemented with carbofuran at the rate of 20 μg g^{-1}. The percentage recovery given is the average of three replicates.

TABLE 3
Degradation of Carbofuran in a Mineral Salts Medium by Pure Cultures of Bacteria

Incubation (days)	*Carbofuran recovered (%)*[a]					
		Inoculated with bacterial suspensions				
	Uninoculated	Pseudomonas stutzeri	Bacillus pumilis	*B1*[b]	*B2*[c]	*B3*[d]
0	94·7	94·7	94·7	94·7	94·7	94·7
10	84·5	67·5	73·5	72·3	58·6	61·1
15	79·5	55·6	61·6	47·8	50·6	49·0
20	76·4	38·4	53·7	40·6	40·2	38·3
30	75·0	1·4	0·6	35·0	11·3	20·0

[a] The mineral salts medium was supplemented with carbofuran at the rate of 20 μg ml^{-1}. The percentage recovery given is the average of three replicates.
[b] Unidentified bacterium No. 1.
[c] Unidentified bacterium No. 2.
[d] Unidentified bacterium No. 3.

This result indicates that enrichment cultures and pure bacterial cultures from non-flooded soil are also capable of enhancing carbofuran degradation.

3.3 Carbofuran degradation by a mixture of pure bacterial cultures

Though 97% loss of carbofuran was observed in a medium inoculated with soil suspensions from carbofuran-retreated soil within 10 days, none of the pure isolates was found to enhance the degradation of carbofuran significantly during that period. Therefore an experiment was conducted to find out whether any synergistic interaction existed between all the pure isolates in enhancing the degradation of carbofuran in their combined presence. The seven pure cultures were mixed together and inoculated into carbofuran-supplemented medium and incubated at room temperature. The mixture of cultures was found to enhance the degradation of carbofuran significantly, effecting 96% loss after 10 days (Table 4). Since only 26–42% carbofuran was lost from media inoculated with individual cultures (Table 3) within 10 days, 96% loss during this period indicates that the bacterial isolates have interacted synergistically in enhancing the degradation of carbofuran. In an earlier study[13] it was found that *Pseudomonas* sp. synergistically increased the degradation of carbofuran by *Arthrobacter* sp. This study shows that micro-organisms present in the soil can interact synergistically to enhance carbofuran degradation to a great extent.

TABLE 4
Degradation of Carbofuran in a Mineral Salts Medium by Mixture of Seven Pure Cultures of Bacteria

Incubation (days)	*Carbofuran recovered (%)*[a]	
	Uninoculated	*Inoculated with mixture of seven pure cultures*
0	95·4	95·4
10	87·6	4·0
15	83·3	1·0

[a] The mineral salts medium was supplemented with carbofuran at the rate of 20 μg ml^{-1}. The percentage recovery given is the average of three replicates.

ACKNOWLEDGEMENT

The authors thank Director, IIHR, for providing facilities to carry out this work.

REFERENCES

1. Reddy, P. P., Nematode management in vegetable crops. In *Advances in Horticulture, vol. 6—Vegetable Crops: Part 2*, ed. K. L. Chadha & G. Kalloo. Malhotra Publishing House, New Delhi, 1993, pp. 829–58.
2. Tandon, P. L., Insects and mite pests of tropical fruits. In *Advances in Horticulture, vol. 3—Fruit Crops: Part 3*, ed. K. L. Chadha & G. Kalloo. Malhotra Publishing House, New Delhi, 1993, pp. 1527–55.
3. Venkateswarlu, K., Gowda, T. K. S. & Sethunathan, N., Persistence and biodegradation of carbofuran in flooded soils. *J. Agric. Food Chem.*, **28** (1977) 533–6.
4. Rajagopal, B. S., Brahmaprakash, G. P., Reddy, B. R., Singh, U. D. & Sethunathan, N., Effect and persistence of selected carbamate pesticides in soils. *Res. Rev.*, **93** (1984) 1–199.
5. Rajagopal, B. S., Brahmaprakash, G. P. & Sethunathan, N., Degradation of carbofuran by enrichment cultures and pure cultures of bacteria from flooded soils. *Environ. Pollut.* Ser. A, **36** (1984) 61–74.
6. Racke, K. D. & Coats, J. R., Enhanced degradation and the comparative fate of carbamate insectides in soils. *J. Agric. Food Chem.*, **36** (1988) 1067–72.

7. Read, D. C., Enhanced microbial degradation of carbofuran and fensulfothion after repeated application to acid mineral soil. *Agric. Ecosystems Environ.*, **10** (1983) 37–46.
8. Read, D. C., Accelerated microbial degradation of carbofuran in soil from previously treated fields. *Agric. Ecosystems Environ.*, **15** (1986) 51–61.
9. Bruce, C. L., James, C. M., Robert, C. H. & Glenn, H. F., Determination of carbosulfan and carbofuran residues in plant, soil and water by Gas Chromatography. *J. Agric. Food Chem.*, **31** (1983) 220–30.
10. Sethunathan, N., Diazinon degradation in submerged soil and rice-paddy water. In *Fate of Organic Pesticides in the Aquatic Environment*, ed. R. F. Gould. Adv. in Chem. Ser., **111**, American Chemical Society, Washington DC, 1972, 244–55.
11. Chapman, R. A., Harris, C. R. & Harris, G., Observation on the effect of soil type, treatment intensity, insecticide formulation, temperature and moisture on the adaptation and subsequent activity of biological agents associated with carbofuran degradation in soil. *J. Environ. Sci. Health*, **21B** (1986) 125–41.
12. Rajagopal, B. S., Rao, V. R., Nagendrappa, G. & Sethunathan, N., Metabolism of carbaryl and carbofuran by soil enrichment cultures and bacterial cultures. *Can. J. Microbiol.*, **30** (1984) 1458–66.
13. Singh, N., Sahoo, A., Mishra, D., Rao, V. R. & Sethunathan, N., Synergistic interaction between two bacterial isolates in the degradation of carbofuran. *Biodegradation*, **4** (1983) 115–23.